## ACCELERATED PUBLICATION

# Fibulin-5 interacts with fibrillin-1 molecules and microfibrils

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Fibulin-5 plays an important role in elastic fibre formation *in vivo*. We have investigated the molecular interactions between fibulin-5 and components of fibrillin-rich microfibrils which form a template for elastin. Fibulin-5 interacted in a dose-dependent manner with a fibrillin-1 N-terminal sequence and with tropoelastin, but not with MAGP-1 (microfibril-associated glycoprotein-1) or decorin. Fibulin-5 did not inhibit interactions between fibrillin-1 N- and C-terminal fragments, or fibrillin-1 interactions with tropoelastin. Fibulin-5 may provide a link between tropoelastin and microfibrils in the pericellular space during elastic fibre assembly.

Key words: elastic fibre, fibulin-5, fibrillin-1, microfibril, microfibril-associated glycoprotein-1 (MAGP-1), tropoelastin.

## INTRODUCTION

Fibulin-5 (molecular mass 56 kDa) is a modular calcium-binding extracellular matrix protein that is predominantly expressed in developing arteries [1–4]. Adult blood vessel cells also express it at low levels, but its expression is markedly enhanced in response to vascular injury and in atherosclerotic plaques [3], as well as in lung vascular endothelial cells [4]. Fibulin-5 contains an N-terminal cbEGF (calcium-binding epidermal growth factor)-like domain with an Arg-Gly-Asp cell-adhesion motif, five contiguous cbEGF-like domains and a C-terminal fibulin-type module. It has recently been shown to play important roles in elastic fibre formation, in linking elastic fibres to cells, in vascular cell signalling and migration, and in regulating superoxide dismutase in blood vessels [5–9]. Fibulin-5-deficient mice exhibit defective elastic fibre formation, leading to marked elastinopathy throughout the body, causing loose skin, vascular abnormalities associated with loss of compliance and severe emphysema [6,7]. Mutations in the fibulin-5 gene also cause cutis laxa and age-related macular degeneration in humans [10–12].

In the present study, we have found that recombinant human fibulin-5 interacts with an N-terminal sequence of human fibrillin-1 and with fibrillin-rich microfibrils, but not with microfibril-associated molecules, MAGP-1 (microfibril-associated glycoprotein-1) or decorin. We localized the fibulin-5-binding region on microfibrils, and determined the binding kinetics for its interactions with fibrillin-1 and tropoelastin. Our data support a role for fibulin-5 interactions with fibrillin-1 as a link between tropoelastin and microfibrils in the pericellular space during elastic fibre assembly.

## EXPERIMENTAL

### Recombinant human fibulin-5

Full-length recombinant human fibulin-5 (Figure 1A) was expressed and purified in milligram amounts using a mammalian episomal expression system and 293-EBNA (Epstein–Barr nuclear antigen) cells. The pCEP-pu/AC7 vector was modified by incorporation of an N-terminal $His_6$ tag following the signal peptide to allow rapid fragment purification by nickel chromatography [13]. Fibulin-5 was monomeric on SDS/PAGE in the presence or absence of 10 mM dithiothreitol (Figure 1C) and by size fractionation on Superdex 200 HR 10/30 columns, and was N-glycosylated as tested by PNGase F (peptide N-glycosidase F) (results not shown).

### Recombinant fibrillin-1, MAGP-1 and tropoelastin

Production of the recombinant human fibrillin-1 fragments, human MAGP-1 and human tropoelastin has been described previously [13–15] (Figure 1B).

### Solid-phase binding assays

These studies utilized recombinant human fibrillin-1 peptides PF1, PF2, PF3, PF5, PF7, PF8, PF11, PF12, PF13, fibulin-5, MAGP-1, tropoelastin and purified decorin (from Professor T. E. Hardingham, Faculty of Life Sciences, Manchester University, Manchester, U.K.).

Soluble ligands (fibulin-5, fibrillin-1 fragments PF1, PF2 and PF7) were biotinylated, as described in [13,15]. Flat-bottomed microtitre plates (Thermo Labsystems, Franklin, MA, U.S.A.) were coated with fibrillin-1 fragments, fibulin-5, tropoelastin, MAGP-1 or decorin at either 5 $\mu$g/ml or 100 nM in TBS (Tris-buffered saline; 50 mM Tris/HCl, pH 7.4, and 0.1 M NaCl) overnight at 4 °C. BSA blocking, washing, binding and detection steps were carried out as described previously [13,15]. Soluble biotinylated protein dilutions of 0–25 $\mu$g/ml for binding curves, and 100 nM for subsequent analyses, were used. All assays were performed in triplicate and were repeated at least twice to confirm observed results. Dissociation constant ($K_d$) values for dose-dependent interactions were calculated using non-linear regression with one-site binding (hyperbola) (GraphPad Prism 2.0) [13,15]. Data were statistically analysed using unpaired Student's $t$ tests (Microsoft Excel 2000). Results are statistically significant when the $P$ value is $< 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). All data are shown as mean values $\pm$ S.E.M. Calcium dependencies of binding interactions were investigated following ligand

Abbreviations used: cbEGF, calcium-binding epidermal growth factor; LOX, lysyl oxidase; MAGP-1, microfibril-associated glycoprotein-1; STEM, scanning transmission electron microscopy; TBS, Tris-buffered saline.

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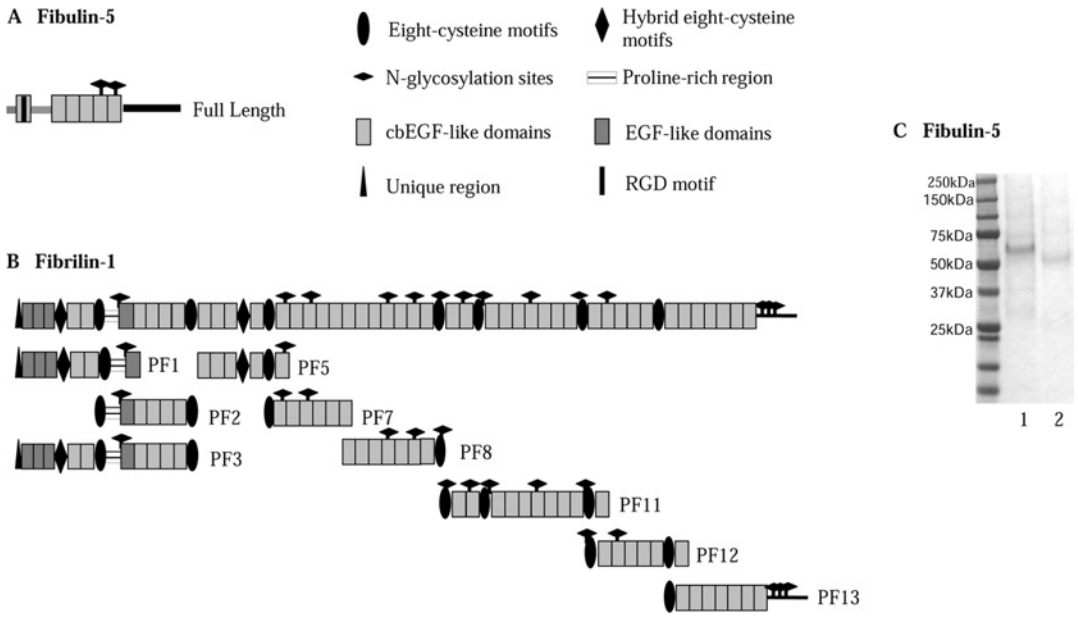


**Figure 1 Domain structures of human fibulin-5 and fibrillin-1**

(**A**) Full-length fibulin-5. (**B**) Full-length fibrillin-1, and the recombinant fragments used in the present study. (**C**) SDS/PAGE gel showing Coomassie-Blue-stained recombinant fibulin-5 under reducing (lane 1) and non-reducing (lane 2) conditions. Molecular-mass markers (sizes given in kDa) are shown in the left-hand lane.

attachment to the wells, by addition of 10 mM EDTA to soluble ligand solutions, as described in [15]. Subsequent steps utilized TBS with calcium omitted.

### Inhibition binding assays

Inhibition binding assays were conducted using biotinylation detection, as reported in [15]. To investigate whether fibulin-5 inhibits fibrillin-1 (PF2 and PF7) interactions with tropoelastin [13], wells were coated with recombinant tropoelastin at 100 nM in TBS overnight at 4 °C. Non-specific binding sites were blocked with TBS containing 4 % BSA at room temperature (20 °C) for 2 h. Plates were washed three times with TBS containing 0.1 % BSA. Selected wells were incubated directly with 100 nM fibulin-5 in TBS overnight at 4 °C. The other wells were incubated in buffer alone. Plates were washed again three times in TBS with 0.1 % BSA. All wells were incubated with 100 nM of biotinylated PF2 or PF7 in TBS overnight at 4 °C. After a further three washes, the plates were incubated at room temperature for 10–15 min in a 1:200 dilution of ExtrAvidin–peroxidase conjugate. Bound protein was quantified, after four more washes, with the same colorimetric assay, using ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)] solution. Plates were read at a wavelength of 405 nm. All experiments were done in quadruplicate wells. Non-specific binding of biotinylated PF2 or PF7 to BSA-blocked wells was also recorded.

Similar inhibition assays were performed to investigate whether fibulin-5 inhibits interactions between fibrillin-1 N-terminal (PF1) and C-terminal (PF13) fragments [15]. In these experiments, PF13 (100 nM) was absorbed on to the wells, and selected wells were pre-incubated with fibulin-5 (100 nM). All wells were then incubated with biotinylated PF1 (100 nM). A positive control involved coating wells directly with fibulin-5 before incubation with biotinylated PF1. Non-specific binding of biotinylated PF1 to BSA-blocked wells was also recorded.

### BIAcore 3000 kinetic analysis of molecular interactions

For kinetic binding studies of tropoelastin and fibulin-5 by surface plasmon resonance, a BIAcore biosensor was used (BIAcore 3000; BIAcore AB, Uppsala, Sweden). Tropoelastin in 10 mM sodium acetate, pH 5.2, was immobilized on to CM5 sensor chips by amine coupling using 1-ethyl-3(3-dimethylaminopropyl)-carbodi-imide hydrochloride, *N*-hydroxysuccinimide and ethanolamine-HCl, as described in [13], giving approx. 13 500 resonance units. Subsequent binding experiments were performed in 10 mM Hepes, pH 7.4, 0.1 M NaCl, 1 mM $CaCl_2$ and 0.005 % surfactant P20 (designated HBS-Ca).

Kinetic analysis used tropoelastin-bound chips and fibulin-5 as the analyte. Fibulin-5 was injected at concentrations in the range 1–8 $\mu$g/ml at a flow rate of 30 $\mu$l/min for 3 min. After 10 min of dissociation, the chip was regenerated in HBS-Ca containing 0.5 mM NaCl for 60 s and then stabilized for 30 min using HBS-Ca, before the next injection was carried out. Analyte was simultaneously passed over a blank-capped flow cell, and this baseline was subtracted from the experimental flow cell. After subtraction of each response value from the blank cell, association and dissociation rate constants were determined by separate $k_{ass}/k_{diss}$ fittings of the binding and dissociation curves and using global data analysis. All curves were fitted using the 1:1 Langmuir association/dissociation model (BIAevaluation 4.1; BIAcore AB). Statistical analysis of the curve fitting at both dissociation and association phases was expressed as $\chi^2$ values. In each case, $\chi^2$ values were $< 10$.

We were unable to conduct BIAcore analysis of fibulin-5 interactions with fibrillin-1 fragments because these molecules did not significantly interact using CM5 or streptavidin chips [13,15]. However, $K_d$ values could be calculated from solid-phase binding assay data, using GraphPad Prism 2.0 (see above).

### STEM (scanning transmission electron microscopy) mass mapping of fibulin-5 binding to microfibrils

Microfibrils were extracted from human ciliary zonules, as described in [16]. Following overnight incubation with and without fibulin-5 (5 ng/$\mu$l in HBS-Ca), unstained microfibrils were examined by dark-field STEM in a Tecnai 12 TWIN electron microscope using a nominal magnification of 160 000×.

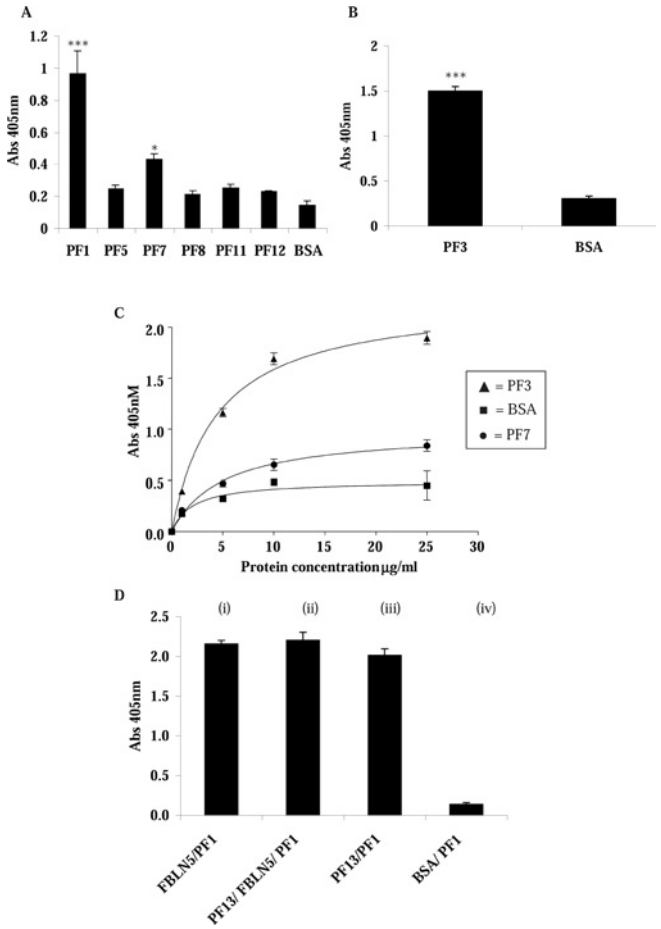


**Figure 2 Interactions of fibulin-5 with fibrillin-1**

(**A**) Immobilized fibrillin-1 fragments (100 nM) or BSA were incubated with 100 nM biotinylated full-length fibulin-5 at 4 °C. (**B**) Immobilized fibrillin-1 fragment PF3 (100 nM) or BSA was incubated with biotinylated full-length fibulin-5 at 4 °C. (**C**) Immobilized fibrillin-1 fragments PF3 and PF7 (5 $\mu$g/ml), and BSA, were incubated with increasing concentrations (0–25 $\mu$g/ml) of biotinylated fibulin-5 at 4 °C. Using non-linear regression, $K_d$ values were calculated using GraphPad Prism version 2.0. (**D**) Immobilized PF13 (100 nM) was incubated with either soluble fibulin-5 (ii) or buffer (iii), before incubation with biotinylated PF1 (100 nM). As a positive control, immobilized fibulin-5 (100 nM) was incubated with biotinylated PF1 (100 nM) (i). BSA-coated wells were incubated with biotinylated PF1 (100 nM) (iv). Under each bar, the 'immobilized' molecule (PF13 or fibulin-5) is listed first, soluble 'competitor' (fibulin-5) is listed next (for ii only), and the 'biotinylated' soluble ligand (PF1) is listed last in all cases. Results are the means $\pm$ S.E.M. of triplicate values.

Microfibril periodicity, total mass per microfibril repeat and axial mass distributions were determined for bead-to-bead repeats [16].

## RESULTS

### Fibulin-5 binding to fibrillin-1

To determine whether fibulin-5 interacts with fibrillin-1, solid-phase binding assays were conducted with fibrillin-1 protein fragments bound to the wells, and binding of soluble biotinylated fibulin-5 was detected. Initial analysis revealed that fibulin-5 bound highly significantly ($P < 0.001$) to N-terminal fibrillin-1 fragments PF1 (encoded by exons 1–11) (Figure 2A) and PF3 (encoded by exons 1–17) (Figure 2B), compared with BSA (Figures 2A and 2B). Fibulin-5 bound less significantly ($P < 0.05$) to central fibrillin-1 fragment PF7 (encoded by exons 24–30). No significant binding was detected to other fibrillin-1 fragments (Figure 2A). Fibrillin-1 fragment PF3 showed strong dose-dependent binding to fibulin-5 (Figure 2C), whereas fibrillin-1 fragment PF7 showed lower dose-dependent binding to fibulin-5 (Figure 2C). The $K_d$ for PF3 binding to fibulin-5 was $56.5 \pm 10.4$ nM. The $K_d$ for PF7 binding to fibulin-5 was $212 \pm 60.6$ nm. We have shown previously that fibrillin-1 N- and C-terminal fragments (PF1 and PF13 respectively) interact with each other [15]; this interaction may be critical for microfibril assembly. However, fibulin-5 did not inhibit this interaction (Figure 2D).

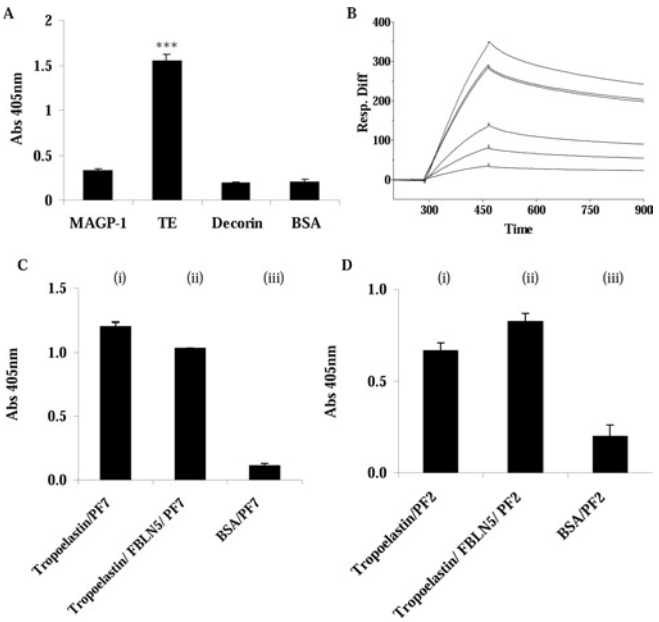


**Figure 3 Interactions of fibulin-5 with immobilized MAGP-1, tropoelastin and decorin**

(**A**) Immobilized MAGP-1, tropoelastin (TE) or decorin (5 $\mu$g/ml) was incubated with biotinylated fibulin-5 (5 $\mu$g/ml) at 4 °C. (**B**) For BIAcore 3000 analysis, fibulin-5 was injected over tropoelastin-immobilized CM5 chip surfaces. The sensorgram shows five different analyte concentrations: 1, 2, 4, 6 (duplicated) and 8 $\mu$g/ml. Resp. Diff (response difference) shows the difference between experimental and control flow cells in response units (RU). Time is shown in s. (**C**) and (**D**) Immobilized tropoelastin (100 nM) was incubated with either soluble fibulin-5 (100 nM) (ii) or buffer (i), before incubation with biotinylated PF7 (**C**) or PF2 (**D**) (both at 100 nM). BSA-coated wells were incubated with biotinylated PF7 (**C**) or PF2 (**D**) (100 nM) (iii). Under each bar, the 'immobilized' molecule (tropoelastin) is listed first, soluble 'competitor' (fibulin-5) is listed next (for ii only), and the 'biotinylated' soluble ligand (PF7 or PF5) is listed last in all cases. Results are the means $\pm$ S.E.M. of quadruplicate values.

### Fibulin-5 binding to other elastic fibre molecules

Solid-phase assays were used to investigate whether fibulin-5 binds to tropoelastin, MAGP-1 or decorin. Fibulin-5 showed significant binding to tropoelastin (Figure 3A), but not to MAGP-1 or decorin (Figure 3A). Fibulin-5 did not block the interactions of fibrillin-1 (PF2 or PF7 respectively) with tropoelastin (Figures 3C and 3D). There was no significant difference in fibulin-5 binding to tropoelastin in the absence or presence of 10 mM EDTA (results not shown).

### BIAcore 3000 kinetic analysis of fibulin-5 binding to tropoelastin

We have shown previously that tropoelastin binds the fibrillin-1 PF2 fragment and overlapping PF5 and PF7 fragments [13]. In the present study, we determined fibulin-5 binding affinity for tropoelastin, by surface plasmon resonance (Figure 3B). Kinetic analysis revealed that the association ($k_{ass}$) and dissociation ($k_{diss}$) rate constants of the binding interaction of fibulin-5 to tropoelastin were $(40.4 \pm 16.9) \times 10^3\ M^{-1} \cdot s^{-1}$ and $(6.3 \pm 0.4) \times 10^{-4}\ s^{-1}$ respectively, and the dissociation constant ($K_d$) was

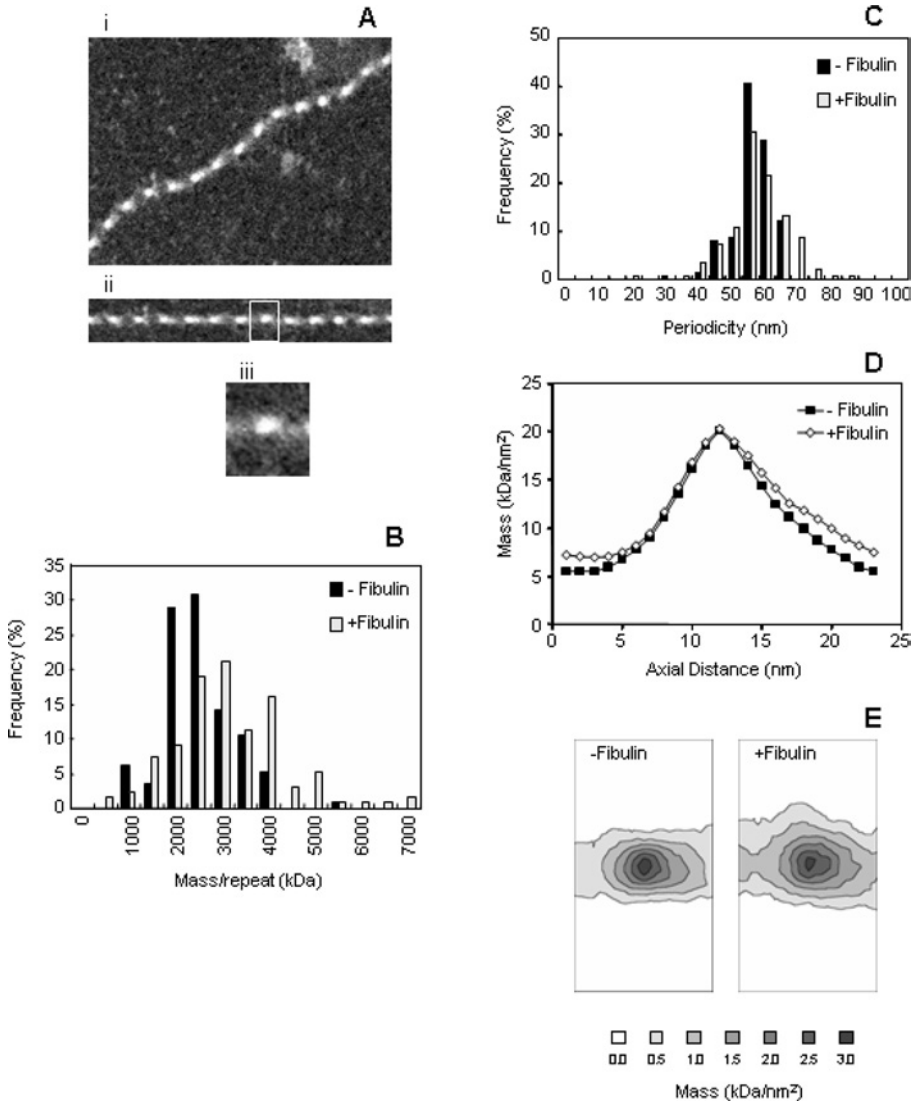


**Figure 4 STEM mass mapping of fibrillin-rich microfibrils**

(**A**) (i) Dark-field STEM image of a fibrillin-rich microfibril incubated with fibulin-5 (465 nm × 350 nm). (ii) Straightened region extracted from image (**A**, i) using the Straighten plug-in written for Image J (a public domain image analysis program developed at National Institutes of Health and available on the internet at http://rsb.info.nih.gov/ij/). (iii) Single repeating unit extracted from (**A**, ii) (57 nm × 107 nm). Mass per repeat (**B**), microfibril periodicity (**C**), mean axial mass distributions (**D**) and mean repeat mass contour maps (**E**) for fibrillin microfibrils with or without fibulin-5 are also shown.

$40.6 \pm 11.6$ nM. BIAcore analysis of fibulin-5 interactions with fibrillin-1 was not possible, as these molecules did not bind the chips significantly.

### STEM mass mapping of fibulin-5 binding to microfibrils

STEM mass analysis of dark-field microfibril images (Figure 4A) revealed that, following incubation with full-length fibulin-5, there was a significant increase in mean microfibril mass from 2252 kDa to 2868 kDa (Student's $t$ test; $P < 0.0001$) (Figure 4B). No significant changes were seen in mean microfibril periodicity (Figure 4C). The additional mass was mapped within the shoulder and interbead regions (Figure 4D and 4E).

## DISCUSSION

It has become evident that fibulin-5 plays critical roles in vascular biology [1–7,9,17]. In particular, it is necessary for functional elastic fibre deposition, a complex hierarchical process involving a number of molecules. Fibrillin-rich microfibrils act as a template for tropoelastin deposition [18], and tropoelastin interactions with fibrillin-1 may be stabilized by transglutaminase [13]. Tropoelastin accretion is accompanied by lysyl-derived cross-linking catalysed by LOX (lysyl oxidase) enzymes. Fibulin-5 can bind tropoelastin directly [6] (Figure 3), and may interact with LOXL1 [19]. Fibulin-5 expression is regulated by tropoelastin [20] and by TGF$\beta$ (transforming growth factor $\beta$) [21]. It was reported that fibulin-5 does not bind the N- or C-terminal halves of fibrillin-1 [6]. However, there are significant fibulin-5 alterations in tight skin (Tsk) mice which express a mutant fibrillin-1 gene, associated with disorganized elastic fibres [22]. We have screened human fibulin-5 for interactions using a panel of human fibrillin-1 fragments that together encompass full-length fibrillin-1. We found that fibulin-5 binds fibrillin-1 N-terminal fragments and microfibrils, but not MAGP-1 or decorin. We also determined the kinetics of fibulin-5 binding to fibrillin-1 and tropoelastin. Our data suggest that fibulin-5 may provide a link between tropoelastin

and microfibrils in the pericellular space during elastic fibre formation.

Fibulin-5 bound to N-terminal fibrillin-1 fragment PF1, so the binding site within fibrillin-1 must be within the sequence encoded by exons 1–11. Since fibulin-5 binds microfibrils, the fibrillin-1-binding site must be available in the assembled state. While some of the N-terminus may be within the microfibril bead structure, an N-terminal fibrillin-1 epitope for monoclonal antibody 2502 (Chemicon, Temecula, CA, U.S.A.) is accessible to the side of the bead [16]. The proline-rich region localizes to the interbead region, sometimes appearing closer to one bead [23]. This localization concurs with our mapping of fibulin-5 to microfibril shoulder and interbead regions (Figure 4). However, fibrillin-1 epitope localizations are dependent on microfibril morphology, which is profoundly influenced by different surface chemistries [24]. MAGP-1 and decorin may be associated with microfibril beads [25–27]; no fibulin-5 binding here correlates with its inability to interact with MAGP-1 or decorin. Fibulin-5 did not inhibit interactions between the N- and C-terminal regions of fibrillin-1; therefore the fibulin-5-binding site on the N-terminal fibrillin-1 fragment PF1 must be distinct from the PF1 sequence that interacts with the C-terminal fragment PF13. Fibulin-5 is unlikely to block microfibril assembly.

Fibulin-5 has been shown to bind tropoelastin [6] (Figure 3), and we have shown previously that tropoelastin binds to two sites on fibrillin-1 [13]. Our current data show that fibulin-5 does not block interactions between fibrillin-1 and tropoelastin, so fibrillin-1 and fibulin-5 must have separate binding sites on tropoelastin. Ternary complexes of tropoelastin, fibulin-5 and fibrillin-1 may occur, or tropoelastin–fibulin-5 complexes may bind microfibrils. Treatment of soluble fibulin-5 with EDTA in calcium-free assay buffer indicated that calcium did not significantly alter fibulin-5 binding to tropoelastin. This result contrasts with a previous report [6], but this could reflect different binding assays and EDTA chelation conditions.

While fibulin-5 is clearly essential for normal elastic fibre formation [6,7], its precise contribution remains unclear. The present study suggests that pericellular fibulin-5, by interacting with fibrillin-1, may regulate the initial deposition of tropoelastin on to microfibrils before cross-linking to fibrillin-1 by transglutaminase [13]. Further accretion of elastin through homotypic interactions with bound elastin, and stabilization of the elastin ‘core’ by LOX cross-links, could then occur. Such a role for fibulin-5 in elastic fibre formation is consistent with its localization along the surface of elastic laminae [6].

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